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New insight into antiphospholipid syndrome: antibodies to $\beta_2$glycoprotein I-domain 5 fail to induce thrombi in rats

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Abstract

Clinical studies have reported different diagnostic/predictive values of antibodies to domain 1 or 4/5 of β2glycoproteinI in terms of risk of thrombosis and pregnancy complications in patients with antiphospholipid syndrome. To obtain direct evidence for the pathogenic role of anti-domain 1 or anti-domain 4/5 antibodies, we analysed the *in vivo* pro-coagulant effect of two groups of 5 serum IgG each reacting selectively with domain 1 or domain 5 in LPS-treated rats. Antibody-induced thrombus formation in mesenteric vessels was followed by intravital microscopy and vascular deposition of β2glycoproteinI, human IgG and C3 was analyzed by immunofluorescence. Five serum IgG with undetectable anti-β2glycoproteinI antibodies served as controls. All the anti-domain 1 positive IgG exhibited potent pro-coagulant activity while the anti-domain 5 positive and the negative control IgG failed to promote blood clot and vessels occlusion. A stronger granular deposit of IgG/C3 was found on the mesenteric endothelium of rats treated with anti-domain 1 antibodies, as opposed to a mild linear IgG staining and absence of C3 observed in rats receiving anti-domain 5 antibodies. Purified anti-domain 5 IgG, unlike anti-domain 1 IgG, did not recognize cardiolipin-bound β2glycoproteinI while able to interact with fluid-phase β2glycoproteinI. These findings may explain the failure of anti-domain 5 antibodies to exhibit in vivo thrombogenic effect and the interaction of these antibodies with circulating β2glycoproteinI suggest their potential competitive role with the pro-coagulant activity of anti-domain 1 antibodies. These data aim at better defining "really at risk" patients for more appropriate treatments to avoid recurrences and disability.

Introduction

Antiphospholipid syndrome (APS) is a chronic autoimmune disorder characterized by recurrent episodes of vascular thrombosis and adverse pregnancy outcomes in the presence of antibodies to phospholipid-binding proteins (aPL) and occurs either as a primary disease or concomitantly to other connective tissue diseases, particularly systemic lupus erythematosus.[1] Although thrombotic occlusion may affect the vessels of all organs and tissues, deep vein thrombosis in the legs often complicated by pulmonary embolism, and thrombotic occlusion of cerebral and coronary arteries leading to stroke and myocardial infarction respectively are common presentations of the syndrome.[2] This clinical condition is also associated with pregnancy morbidity including fetal loss, pre-eclampsia, pre-term delivery and small for gestational age babies.[3] These are serious complications that affect particularly young people and have both social and economic impacts. The disease may sometimes present as catastrophic syndrome, a more severe form of APS characterized by microthrombosis of small vessels in various organs resulting in multiple organ failure.[4]

Anti-cardiolipin (aCL) and anti-β2glycoprotein I (β2GPI) antibodies and lupus anticoagulant (LA) activity are considered markers of APS and are included among the criteria currently proposed for the classification of this syndrome.[1]

Clinical studies have revealed an increased risk of thrombosis and pregnancy complications in patients with medium to high levels of these antibodies and LA present in their plasma.[5] The triple positivity of these laboratory markers has also been shown to be associated with more severe forms of APS.[5] Conversely, the positivity for a single marker is often associated with a much lower risk for the APS clinical manifestations.[5-9]

It has been widely demonstrated that β2GPI is the main antigen recognized by aPL and the reactivity against the protein has been shown to be responsible for the positivity for aCL and anti-β2GPI assays and in part for the LA phenomenon strongly associated with the APS clinical manifestations.[10]

β2GPI circulates in blood mainly in a circular form and is organized into four domains (D1-4) composed of 60 amino acids with two disulfide bonds and a fifth domain (D5) containing extra 24 amino acids that interacts with anionic phospholipids on the target cells/tissues.[11] Besides the classical diagnostic assays measuring antibodies against whole molecule β2GPI, new tests have recently been developed to detect anti-β2GPI antibody subpopulations reacting with different domains of the protein, particularly the combined domains D4/5 and Domain 1 (D1).[5-7,9,12-14]

In APS patients a large proportion of anti-β2GPI antibodies reacts with D1 and recognizes a criptic epitope (Arg39–Arg43) in the native molecule exposed following its interaction with anionic phospholipids [13,15] or oxidation.[16-18] Antibodies directed against D1 of β2GPI with or without anti-D4/5 antibodies have frequently been found in APS patients associated with an increased risk of thrombosis and pregnancy complications.[7,9,19-24] By contrast, isolated high levels of anti-D4/5 antibodies have been reported in non-APS patients with leprosy, atopic dermatitis, atherosclerosis and in children born to mothers with systemic autoimmune diseases [6] and in asymptomatic aPL carriers and are not associated with either vascular or obstetric manifestations of the APS syndrome.[7,9] This finding prompted some authors to suggest that the ratio between anti-D1 and anti-D4/5 may be a useful parameter for identifying autoimmune APS and for ranking the patients according to their risk to develop the syndrome.[7]

An isolated positivity for anti-D4/5 is a rare condition, which is usually associated with the absence of aCL and/or LA and in the majority of the cases with doubtful APS picture which does not fulfill the classification/diagnostic criteria.[25] The finding that antibodies with this isolated specificity are observed mainly in the absence of clinical manifestations of hypercoagulable states has suggested that they may not be involved in thrombus formation.

While the *in vivo* pathogenic role of aPL has been demonstrated for those directed against the whole molecule and against D1 of β2GPI using animal models of thrombosis developed in rats and mice, [26-28] direct evidence that antibodies to D4/5 do not play an *in vivo* pathogenic role in blood clotting is presently lacking nor is it clear whether they are able to interact with soluble or surface bound β2GPI. Data will be presented indicating that the antibodies are ineffective in causing blood clot due to their failure to recognize bound β2GPI.

Methods

Serum source

Two groups of anti-β2GPI positive sera[7,27] containing isolated antibodies to either D1 or D4/5 domains[6,7] and control sera with undetectable anti-β2GPI antibodies were analysed. All samples were also tested for aCL antibodies[7] and LA activity.[29] The anti-D1 positive sera were obtained from APS patients.[1] The sera were collected after obtaining informed consent and the IgG were purified by a Protein G column (HiTrap Protein G HP, GE Healthcare) as described.[27] The local Istituto Auxologico Italiano ethical committee approved the study.

Purification of β2GPI and generation of recombinant domains D4 and D5

The purification of human β2GPI from pooled normal sera[27,30] and the generation of D4 and D5 domains[12,31] have been published. Sequence analysis was performed as described[32] and compared to the published sequence of β2GPI.[33] The fine specificity against D4 or D5 was investigated by ELISA.[27]

Animal model

An in vivo model of antibody-induced thrombus formation was established in male Wistar rats (270-300g) and kept under standard conditions in the Animal House of the University of Trieste, Italy as previously reported in details.[26] The in vivo procedures were performed in compliance with the guidelines of European (86/609/EEC) and Italian (D.L.116/92) laws and were approved by the Italian Ministry of University and Research and the Administration of the University Animal House. This study was conducted in accordance with the Declaration of Helsinki. Details reported in the *Supplementary Methods*.

Immunofluorescence analysis

The mesenteric tissue was collected from rats at the end of the in vivo experiment.[26] Deposits of β2GPI were analyzed using the biotinylated monoclonal antibody MBB2 and FITC-labeled streptavidin (Sigma-Aldrich).[27] IgG and C3 were detected using FITC-labeled goat anti-human IgG (Sigma-Aldrich) and goat anti-rat C3 (Cappel/MP Biomedicals) followed by FITC-labeled rabbit anti-goat IgG (Dako), respectively. The slides were examined using a DM2000 fluorescence microscope equipped with DFC 490 photo camera and the Application Suite software (Leica).

Antibody binding assays

Different concentrations of β2GPI were added to CL-coated plates and the reactivity of IgG with CL-bound β2GPI was measured.[7] The interaction of IgG with soluble β2GPI was evaluated by incubating IgG with increasing concentrations of β2GPI or BSA as unrelated antigen for 1 hour at 37°C followed by overnight incubation at 4°C in a rotator. The samples were centrifuged at 3,000 g for 5 min at room temperature and the residual un-complexed antibodies were tested using β2GPI-coated plates (Combiplate EB, Labsystems) as described.[7] Details reported in the *Supplementary Methods*.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 for Windows. The domain reactivity of the anti-β2GPI D4/5 positive sera was expressed as mean±SD and analyzed with the paired Student's t test. Data from *in vivo* thrombus formation were compared by Dunnett's test. The interaction between IgG and β2GPI bound to CL was analyzed with the Kruskall-Wallis with Dunn's *post-hoc* test. The interaction between IgG and soluble β2GPI was expressed as median and interquartile range and analyzed with the 2way repeated measure ANOVA with Sidak's *post-hoc* test. Probabilities of ≤0.05 were considered statistically significant.

Results

aPL profile of the serum samples

Anti-β2GPI IgG titers were comparable in the anti-D4/5 and anti-D1 positive samples (1.04 ± 0.26 OD and 1.46 ± 0.48 OD, mean ± SD, respectively). The isolated anti-D4/5 positive samples displayed anti-D4/5 level of 50.67± 9.86 AU (mean ± SD) while they were negative for aCL (<10 GPL) and LA. The isolated anti-D1 positive samples showed anti-D1 level of 75.36 ± 17.15 AU (mean ± SD), high titers of IgG aCL (124.4 ± 46.9 GPL, mean ± SD) and displayed LA activity. Control samples were negative in all the assays. The purified IgG fractions maintained the antigen specificity shown in the whole serum. Clinical and serological data of all the included subjects/patients are reported in *Supplementary Table 1*.

Fine epitope-specificity of antibodies to D4/5

The IgG against D4/5 used in this study were selected for their ability to react with the combined domains obtained from INOVA Diagnostics, but it was unclear whether they recognized one or the other domain or both. To clarify this point, we assessed the reactivity of serum IgG towards recombinant D4 and D5 domains. The amino acid sequences of the two domains are reported in *Supplementary Figure 1*. The results presented in Figure 1 clearly show that all the anti-D4/5 reacted with D5 and did not recognize D4. The difference in the reactivity of the various serum IgG towards D4/5 is essentially similar to that observed in their reaction with D5.

Antibodies to D5 fail to cause thrombus formation in vivo

To evaluate the pro-coagulant activity of sera containing antibodies to different domains of β2GPI,

two groups of serum IgG positive for either D1 or D5 domains were analyzed for their ability to induce thrombus formation followed in vivo by intravital microscopy. IgG from sera negative for antibodies to β2GPI served as a control group. As shown in Figure 2, all anti-D1 positive IgG induced blood clots that started to be seen 15 min after serum infusion. Their number progressively increased to reach the highest value after 1 hour and was maintained thereafter up to 90 min. Thrombus formation was associated with vascular occlusion that resulted in marked decrease, and, in some vessels, in complete blockage of blood flow. Conversely, the anti-D5 positive IgG did not exhibit pro-coagulant activity and failed to cause reduced blood flow. The latter results were not statistically different from those of anti-β2GPI negative blood donors at each time point. On the contrary, the data of anti-D1 IgG were statistically different from those of anti-β2GPI negative samples at all times starting from 15 minutes of analysis with a $P < 0.05$.

Antibodies to D5 fail to interact with surface-bound β2GPI

Having observed absence of intravascular coagulation in rats that had received anti-D5 positive IgG, we decided to investigate whether this was due to the inability of the antibodies to interact with endothelium-bound β2GPI. To this end, samples of ileal mesentery were analyzed for the presence of β2GPI, human IgG and C3. As expected from our previous findings,[30] β2GPI was detected on the vessel endothelium of rats primed with LPS (Figure 3), while totally absent in unprimed animals (data not shown). Search for IgG and C3 revealed marked granular deposits of both proteins on endothelial cells of rats treated with anti-D1 IgG, while a milder linear staining for IgG and absence of C3 were observed in rats receiving anti-D5 IgG (Figure 3). The animals treated with anti-β2GPI negative sera showed negligible staining for IgG and undetectable C3 (Figure 3). Since several molecules other than β2GPI are expressed on the endothelial cell surface and represent potential targets for human IgG, we set out to determine whether the fluorescence was due to the IgG specifically against β2GPI. To do this, we set up a β2GPI-dependent CL assay in which the β2GPI supplementation was carried out by adding human purified β2GPI at increasing concentrations instead of fetal calf serum. The system allowed us to test the IgG reactivity with β2GPI added at different concentrations to the CL-plates. As shown in Figure 4, the anti-D1 IgG reacted with the β2GPI molecule most likely by recognizing the D1 epitope exposed on the β2GPI molecule following its binding to cardiolipin. The IgG level detected in the assay varied in different patients and was related to the concentration of β2GPI used to coat cardiolipin. In contrast, anti-D5 IgG failed to interact with cardiolipin-bound β2GPI even at the highest concentration of β2GPI,

suggesting that D5 domains were not accessible to the antibodies under these experimental conditions. Like the anti-D5 antibodies, the IgG from control sera were negative in the assay.

Antibodies to D5 interact with soluble β2GPI

Electron microscopy studies have revealed that β2GPI adopts a circular form in plasma maintained by the interaction of D1 with D5.[34] This special conformation prevents the access of autoantibodies to hidden epitopes on D1[19] and predicts the presence of cryptic epitopes on D5, though this has not been formally proven.[35]

We first decided to examine the in vivo interaction of the antibodies with circulating β2GPI and the effect of this interaction on β2GPI bound to vascular endothelium. To this purpose, the in vivo model was slightly modified administering IgG intraperitoneally followed 15 hours later by LPS given by the same route. This approach would allow sufficient time to the antibodies to react with the target antigen prior to the binding of β2GPI to vascular endothelium promoted by LPS. The IgG from two sera with relatively high levels of antibodies to D1 and D5 respectively, and from an anti-β2GPI negative serum were tested and the amount of vascular deposits of β2GPI and IgG was evaluated. As expected, the rat treated with anti-D1 developed endovascular thrombi associated with deposition of IgG, both of which were undetectable in animals that received anti-D5 positive or anti-β2GPI negative IgG (Figure 5). Analysis of the ileal mesentery showed that β2GPI was present on the vascular endothelium of the animals that received the three IgG fractions with no clear difference in the staining intensity observed in the rats treated with anti-D5 and anti-D1 IgG (Figure 5).

Since the in vivo data did not provide convincing evidence on the ability of anti-D5 to prevent binding of circulating β2GPI to vascular endothelium, we decided to further investigate this issue using an in vitro inhibition assay. IgG purified from anti-D5 positive, anti-D1 positive or anti-β2GPI negative sera were incubated with increasing concentrations of soluble β2GPI and the residual IgG interacting with β2GPI directly bound to the plate wells were measured. As shown in Figure 6, the amount of IgG anti-D5 free to bind to solid-phase β2GPI after incubation with the soluble molecule decreased compared to that of the IgG incubated with BSA, particularly at higher concentration of soluble β2GPI. In contrast, the level of IgG anti-D1 bound to solid-phase β2GPI following incubation with soluble β2GPI was slightly lower, but not significantly different from that of the IgG incubated with BSA.

Discussion

APS is now recognized as an antibody-dependent and complement-mediated syndrome and antibodies to β2GPI have been identified as important players in thrombus formation in APS patients.[10] Efforts are being made to determine the clinical relevance of antibodies to D1 and D4/5 domains of the molecule detected in these patients. Clinical studies have suggested that antibodies to D4/5, unlike those directed against D1, do not represent a risk factor for thrombosis and pregnancy complications.[7,9,14] The in vivo data presented here focused on the thrombotic aspect of the syndrome and support the clinical observation that the anti-D4/5 antibodies are pathologically irrelevant.

The animal model used in this and in previous studies proved to be an invaluable tool to investigate the ability of the anti-β2GPI antibodies to induce blood clots in rats primed with LPS that provides the first hit followed by the infusion of the antibodies acting as a second hit.[10] As expected, all anti-D1 IgG promoted thrombus formation and vascular occlusion confirming the pathogenicity of these antibodies suggested by the clinical observations. It is possible that LA detected in the plasma of these patients may have also contributed to anti-β2GPI-induced blood clots. However, although β2GPI antibody-dependent LA has been shown to correlate with the increased risk of thrombosis, [13,14,36] evidence supporting the *in vivo* pro-thrombotic activity of LA independent of anti-β2GPI antibody has not been provided yet. Instead, there is good evidence that the antibodies recognizing the D1 domain of β2GPI are directly involved in thrombus formation and vessel occlusion. We have previously shown that a human monoclonal antibody that recognizes D1 induces blood clots and that a CH2-deleted non-complement fixing variant molecule competes with anti-β2GPI antibodies from APS patients and prevents their pro-coagulant activity.[27] A similar inhibitory effect was obtained using recombinant D1 to control the thrombus enhancement activity of aPL in mice.[37]

The *in vi*vo experiments showed that none of the anti-D5 IgG exhibited a prothrombotic activity supporting the observations made in clinical studies that these antibodies are pathologically irrelevant.[7,14] A possible explanation for this finding is the inability of these antibodies to interact with cell-bound β2GPI. In line with this hypothesis we showed that anti-D5 positive IgG fractions were unable to react with β2GPI bound to CL-coated plates in vitro because of the shielding of D5 in the β2GPI molecule bound to CL-coated plate. However, the mild staining for IgG on the endothelium of mesenteric vessels observed in vivo in rats treated with LPS, to promote binding of β2GPI, and anti-D5 IgG did not allow any definite conclusion on this issue. It must be emphasized, however, that the staining intensity varied among different sera and was not related to the level of

antibodies. The linear deposition of IgG on the mesenteric endothelium from rats treated with anti-D5 positive IgG suggests their interaction with antigens constitutively expressed on endothelial cells. This distribution pattern differs from the irregular staining for IgG seen with the anti-D1 positive IgG most likely explained by their reaction with a plasma derived molecule, such as β_2GPI, bound to the endothelial cell surface. The different distribution of anti-D1 and anti-D5 IgG resembles the well-known difference in the granular and linear distribution patterns of IgG observed in the kidney of patients with Systemic Lupus Erythematosus (SLE) and Good-Pasture respectively. The linear pattern of IgG in Good-Pasture is the result of interaction of the antibodies with their target antigen constitutively expressed on the glomerular basement membrane. In contrast, the granular distribution of IgG in SLE is caused by irregular deposition of circulating immune complexes.[38,39] The finding that C3 deposition was undetectable on the vascular endothelium of rats treated with anti-D5 IgG is consistent with the failure of these antibodies to induce thrombus formation. We and others have provided convincing evidence that complement activation is critically involved in the coagulation process induced by anti-β_2GPI IgG and in this study by antibodies to the D1 domain.[26,27,40-43]

The anti-D4/D5 antibodies present in the sera analysed in this study recognized selectively the recombinant D5 domain and are likely to inhibit deposition of β_2GPI on the endothelium by shielding its binding site for the anionic phospholipid on endothelial cells.[44] Our attempt to document *ex vivo* reduced binding of circulating β_2GPI to vascular endothelium of the anti-D5-treated rats was unsatisfactory most likely due to an exceedingly higher level of serum β_2GPI compared to that of injected antibodies *in vivo*. The *in vitro* data obtained under more controlled conditions of IgG and β_2GPI concentrations showed a fluid phase interaction between anti-D5 IgG and soluble β_2GPI, resulting in a significantly reduced reactivity of these antibodies against surface-bound β_2GPI (when the molecule was bound to a plate).

The finding that anti-D5 IgG have no pro-coagulant effect in our in vivo model has important clinical implications suggesting that individuals with isolated presence of these antibodies should not be considered at risk of thrombosis. It should be pointed out, however, that anti-D1 and anti-D5 IgG often co-exist in a large proportion of APS patients, who are likely to be susceptible to anti-D1-dependent thrombus formation. In view of the ability of the anti-D5 IgG to interact with soluble β_2GPI preventing its binding to the target cells, it is tempting to speculate that the anti-D5 IgG may antagonize the pro-coagulant activity of anti-D1 antibodies, depending on the antibody levels. Accordingly, we recently published data indicating that the risk of thrombosis in patients positive for anti-D1 and anti-D4/5 antibodies is reduced if the levels of anti-D4/5 are higher than those of

anti-D1 antibodies.[7,9] Overall our experimental findings fit with the clinical observation, offering new tools for stratifying patients into different risk categories. This would help in better preventing recurrences of the clinical manifestations and avoiding overtreatment, so ultimately improving the patients' quality of life and sparing side effects of treatment.

In conclusion, the data presented in this work indicate that, unlike the anti-D1 positive sera, those containing antibodies against D5 are unable to induce clot formation and vascular occlusion. The failure of the anti-D5 antibodies to promote coagulation is due mainly to their inability to interact with the target epitopes hidden on the surface-bound molecule and possibly to the recognition of native β_2GPI in plasma that may potentially prevent to some extent its binding to the surface of activated endothelial cells. The detection of anti-D5 antibodies in patients with doubtful clinical picture and a single positivity for anti-β_2GPI in the absence of a positive aCL assay may offer a valuable tool for ruling out a definite APS diagnosis and for identifying subjects at lower risk for clinical manifestations.

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Figure legends.

Figure 1. Anti-domain (D) 4/5 antibodies specifically react against domain (D) 5 of β2glycoprotein I (β2GPI). Reactivity of 5 anti-D4/5 positive patient sera (P1-P5) against different recombinant human β2GPI domains: (A) reactivity against the combined D4/5 peptides (■), in an assay produced for research use (QUANTA Lite β2GPI D4/5 ELISA, INOVA Diagnostics) (B) reactivity against the recombinant domains D4 (▭) or D5 (■) antigens separately immobilized on the wells of γ-irradiated polystyrene plates in an in-house ELISA plates. The OD values are expressed as mean ± SD. The data were analyzed with the Student's t test for paired data. The average reactivity against D5 is significantly higher than that against D4 (P = 0.0428).

Figure 2. Anti-domain (D) 5 antibodies fail to induce thrombi in rats. Thrombus formation and vascular occlusion visualized by intravital microscopy in the ileal mesentery of rats that received an intraperitoneal injection of LPS (2.5 mg/kg body weight) followed by the injection into carotid artery of antibodies (10 mg/rat) directed against domain 5 (D5), domain 1 (D1), or anti-β2glycoprotein I (β2GPI) negative (NHS). The number of thrombi (A) and vessel occlusions (B) were evaluated at various time intervals on 3 rats per each serum. The results are expressed as a ratio between the number of thrombi and the number of microvessels examined and as a percentage of occluded microvessels. The data are reported as mean ± SD. (C) Sections of the ileal mesentery showing endovascular thrombi in anti-D1 treated rat and undetectable in the vessels of animals receiving anti-D4/5 positive or anti-β2GPI negative sera. Original magnification 100x. Scale bar 50 μm.

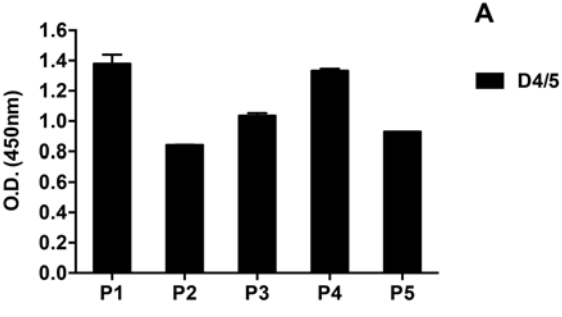
Figure 3. Deposition of β2glycoprotein I (β2GPI), human IgG and C3 on mesenteric vessels of rats treated with antibodies to domain 5 (D5) or domain 1 (D1) of β2GPI. The animals were treated with LPS followed by the injection of antibodies directed against domain 5 (D5), domain 1 (D1), or negative for anti-β2GPI (NHS). Mesenteric tissue samples after 90 min analyzed for vascular deposition of β2GPI, human IgG and C3 by immunofluorescence. Original magnification 200x. Scale bar 50 μm.

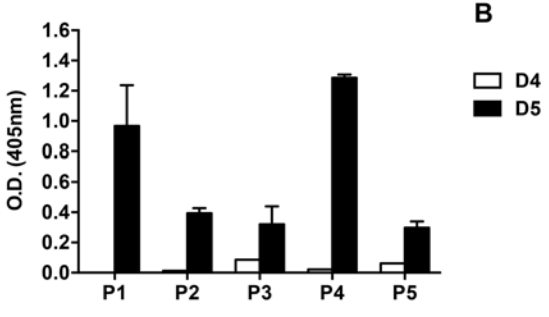
Figure 4. Anti-domain 5 (D5) antibodies fail to interact with β2glycoprotein I (β2GPI) bound to cardiolipin. Reactivity of anti-D5 (aD5) (▭), anti-domain 1 (aD1) (▭) or anti-β2GPI negative (NHS) (▭) antibodies (50 μg/ml) against different concentrations of β2GPI bound to cardiolipin. Binding of IgG to: (A) cardiolipin alone; (B) 1 μg/ml cardiolipin-bound β2GPI; (C) 5 μg/ml

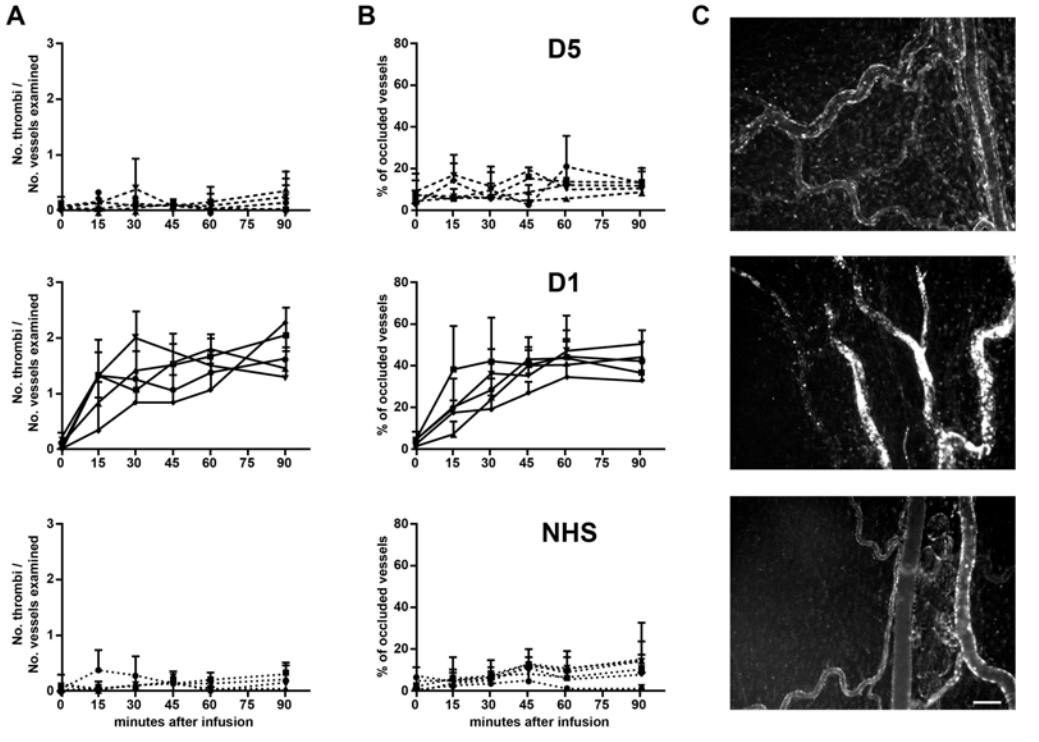
cardiolipin-bound β2GPI; (D) 75 μg/ml cardiolipin-bound β2GPI. The OD values are expressed as median and interquartile range and presented as box plots. *$P < 0.05$.

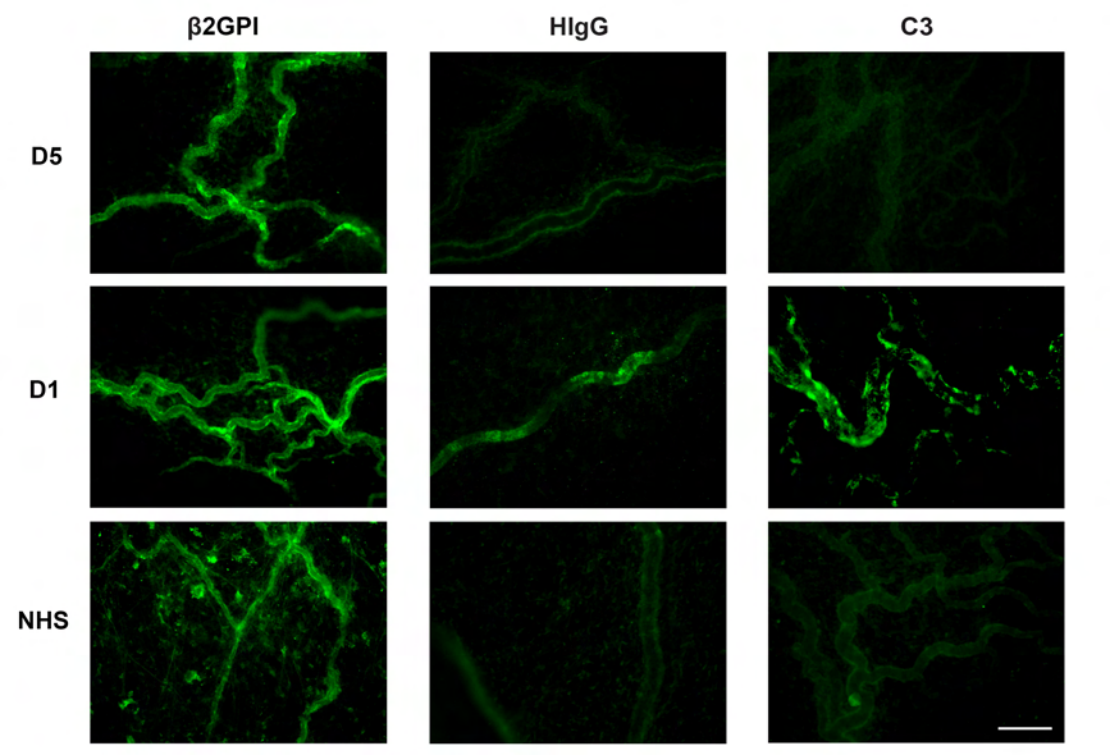
Figure 5. Deposition of β2glycoprotein I (β2GPI) and IgG on mesenteric vessels of rats treated with patients' and controls' serum IgG prior to LPS challenge. The animals were treated with antibodies directed against domain 5 (D5), domain 1 (D1), or anti-β2GPI negative (NHS) (10 mg/rat) before LPS administration (2.5 mg/kg body weight). Mesenteric tissue samples were analyzed for vascular deposition of β2GPI (left panel) and human IgG (central panel). Original magnification for immunofluorescence analysis 200x. Scale bar 50 μm.
Thrombus formation in mesenteric vessels was monitored by intravital microscopy for 90 min and mesenteric tissue was collected at the end of the experiment. Thrombi formed in the vessels are indicated with arrows (right panel). Original magnification 100x. Scale bar 50 μm.

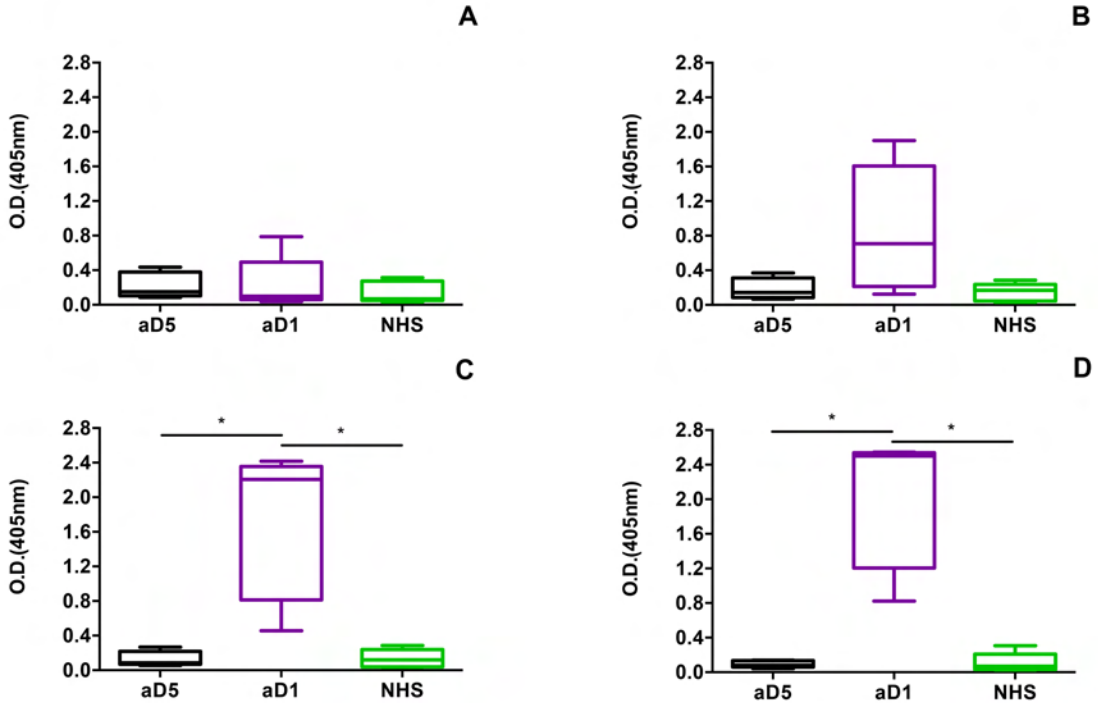
Figure 6. Anti-domain 5 (D5) antibodies interact with β2glycoprotein I (β2GPI) in fluid phase. Reactivity of anti-D5 (aD5), anti-D1 (aD1), or anti-β2GPI negative (NHS) antibodies (50 μg/ml) against purified β2GPI directly coated on ELISA plates, measured after their incubation with (A) 50 μg/ml, (B) 100 μg/ml and (C) 200 μg/ml of purified β2GPI ( ) or BSA ( ) in fluid phase. The OD values are expressed as median and interquartile range and presented as box plots. *$P < 0.05$; **$P < 0.01$.

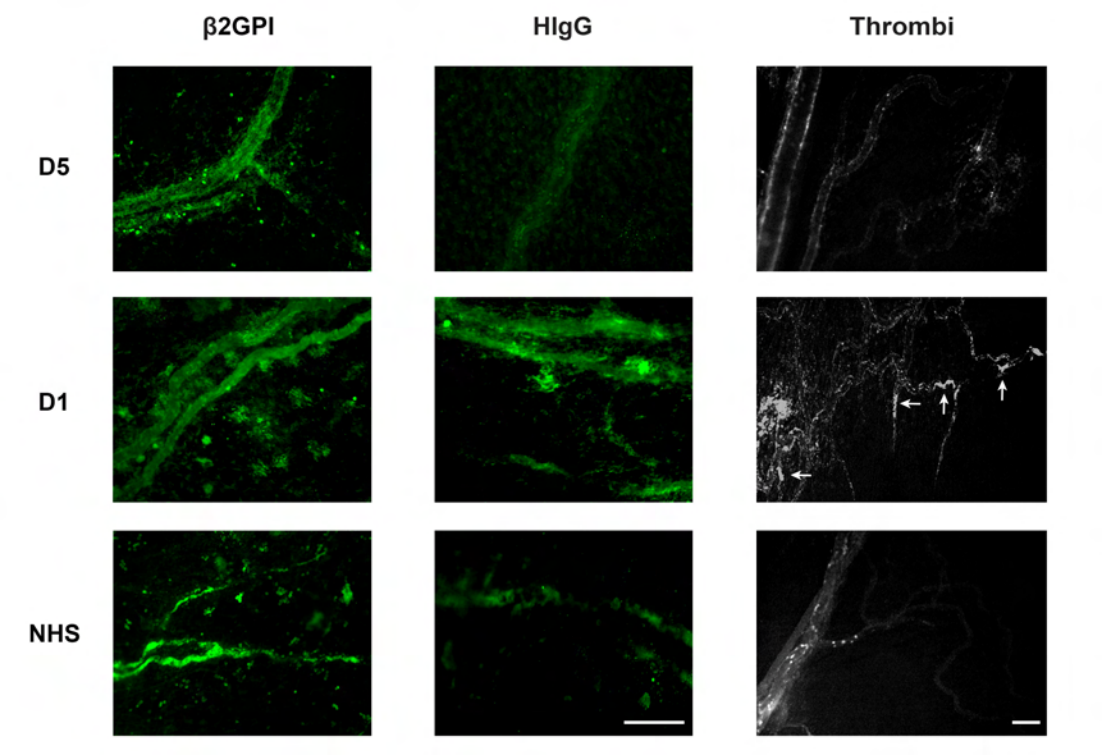

A
D4/5
O.D. (450nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P1
P2
P3
P4
P5

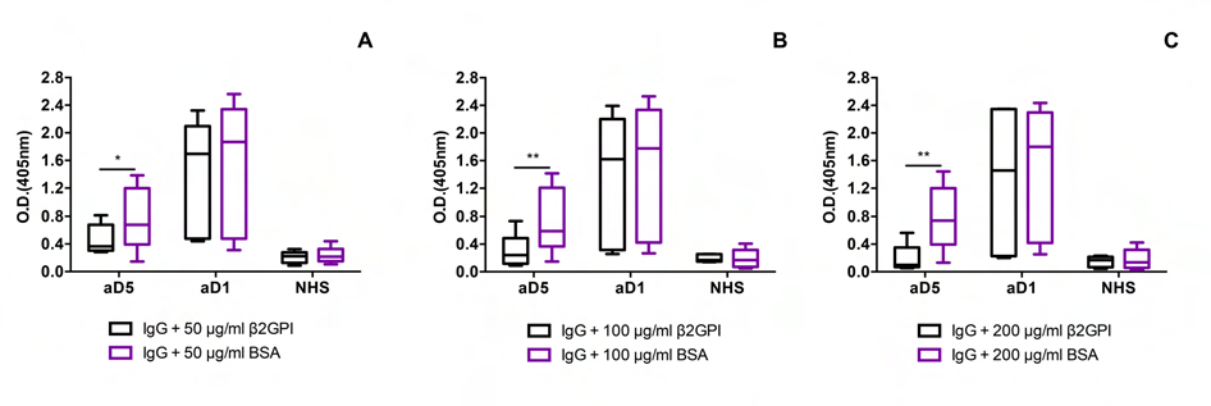

B
D4
D5
O.D. (405nm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P1
P2
P3
P4
P5


A
B
C
D5
D1
NHS
No. thrombi / No. vessels examined
% of occluded vessels
minutes after infusion
0
15
30
45
60
75
90
20
40
80


β2GPI
HIgG
C3
D5
D1
NHS


A
B
C
D
O.D.(405nm)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
aD5
aD1
NHS
*
*


β2GPI
HIgG
Thrombi
D5
D1
NHS


A
B
C
O.D.(405nm)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
*
**
**
aD5
aD1
NHS
IgG + 50 µg/ml β2GPI
IgG + 50 µg/ml BSA
IgG + 100 µg/ml β2GPI
IgG + 100 µg/ml BSA
IgG + 200 µg/ml β2GPI
IgG + 200 µg/ml BSA

Supplementary Methods

Animal models

An *in vivo* model of antibody-induced thrombus formation was established in male Wistar rats (270-300 g) (Envigo, S. Pietro al Natisone, Italy) and kept under standard conditions in the Animal House of the University of Trieste, Italy as previously reported in details.[1] Briefly, the animals received an intraperitoneal injection of lipopolysaccharide (LPS) from Escherichia coli O55:B5 (2.5 mg/kg body weight) (Sigma-Aldrich) 4 hours before general anesthesia. After infusion of Rhodamine 6G (Sigma-Aldrich) into the femoral vein, serum IgG (10 mg/rat) from patients and controls were slowly administered into the carotid artery. For some experiments the protocol was slightly changed and the animals received intraperitoneal injection of IgG 15 hours before general anesthesia followed by the injection of LPS by the same route. Clot formation and partial or complete occlusion of blood vessels were monitored by intravital microscopy and analyzed in at least 5 microvascular areas. The microvasculature was examined using a BX50WI microscope (Olympus, Center Valley, USA), equipped with CCD camera model SensiCam and SensiCam digital converter (PCO). The in vivo procedures were performed in compliance with the guidelines of European (86/609/EEC) and Italian (D.L.116/92) laws and were approved by the Italian Ministry of University and Research and the Administration of the University Animal House. This study was conducted in accordance with the Declaration of Helsinki.

Antibody binding assays

The interaction of IgG with phospholipid-bound β_2GPI was evaluated by coating the wells of 96-well polystyrene plates (Polysorp Immunoplate, Nalge Nunc International) with cardiolipin (50 µg/ml) (Sigma-Aldrich) overnight at 4°C. After blocking the free binding sites with 1% ultrapure BSA (Sigma-Aldrich) in PBS (PBS/BSA), increasing concentrations of purified β_2GPI (1,5,75 µg/ml) were added and left to incubate for 2 hours at room temperature. Free β_2GPI was removed by washing with the blocking buffer and phospholipid-bound β_2GPI was allowed to react with IgG (50 µg/ml) from patients and controls for additional 2 hours at room temperature. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG (Sigma-Aldrich).

The interaction of IgG with soluble β_2GPI was evaluated by incubating patients' and controls' IgG (50 µg/ml) with increasing concentrations (50,100,200 µg/ml) of human purified β_2GPI or BSA as unrelated antigen for 1 hour at 37°C followed by overnight incubation at 4°C in a tube rotator. The samples were centrifuged at 3,000 g for 5 min at room temperature and the residual un-complexed

antibodies were tested using γ-irradiated polystyrene plates (Combiplate EB, Labsystems) directly coated with purified human β_2GPI (10 µg/ml) as previously described.[2]

Supplementary Table 1. Clinical and laboratory characteristics of the patients and controls

Sample ID	Age /sex	Diagnosis	aCL IgG, GPL*	aβ2GPI IgG, OD†	aD1 IgG AU‡	aD4,5 IgG AU‡	LA	AT	VT	PM
P1	35/F	APS non-criteria	0	1.45	9	59	neg	No	No	2EM
P2	56/F	aPL carrier	20	1.10	13	39	neg	No	No	No
P3	29/F	APS non-criteria	4	0.93	24	49	neg	No	No	2EM
P4	36/F	APS non-criteria	3	0.98	15	63	neg	No	No	FGR
P5	60/M	APS non-criteria	10	0.76	6	44	neg	No	SVT	*NA*
P6	34/F	PAPS	88	1.73	69	11	pos	Yes	No	No
P7	51/F	PAPS	155	1.56	78	13	pos	Yes	No	Yes
P8	34/F	SAPS	67	0.62	104	10	pos	No	Yes	No
P9	46/M	PAPS	131	1.68	60	7	pos	Yes	Yes	*NA*
P10	46/F	PAPS	181	1.72	66	15	pos	No	No	Yes
NHS1	39/F	healthy ctrl	6	0.00	0	7	neg	No	No	No
NHS2	27/F	healthy ctrl	5	0.04	4	6	neg	No	No	No
NHS3	43/F	healthy ctrl	8	0.03	13	11	neg	No	No	No
NHS4	42/F	healthy ctrl	10	0.00	16	18	neg	No	No	No
NHS5	34/F	healthy ctrl	6	0.00	0	6	neg	No	No	No

aCL indicates anti-cardiolipin antibodies; aβ2GPI, anti-β2glycoprotein I antibodies; LA, lupus anticoagulant; AT, arterial thrombosis; VT, venous thrombosis; PM, pregnancy morbidity, as defined by Miyakis et al[1]; EM, early miscarriages; FGR, fetal growth retardation; SVT, superficial venous thrombosis; PAPS, primary antiphospholipid syndrome; aPL carrier, antiphospholipid-positive asymptomatic subject; SAPS, secondary antiphospholipid syndrome; ctrl, control; D4/5, β2GPI domains 4/5; D1, β2GPI domain 1; and NA, not applicable. *aCL IgG cut-off 20 GPL; † aβ2GPI IgG cut-off 0.170 OD; ‡Anti-β2GPI D4/5 cut-off 19 AU; and anti-β2GPI D1 cut-off 25 AU

Supplementary Figure 1. Sequences of peptides obtained by trypsin degradation of recombinant domains 4 and 5. The amino acid sequences of domains 4 and 5 are underlined and included in the published sequence of β2GPI.[3]

β2glycoprotein I precursor

1 mispvlilfs sflchvaiag rtcpkpddlp fstvvplktf yepgeeitys ckpgyvsrgg
61 mrkficpltg lwpintlkct prvcpfagil engavryttf eypntisfsc ntgfylngad
121 sakcteegkw spelpvcapi icpppsiptf atlrvykpsa gnnslyrdta vfeclpqham
181 fgndtitctt hgnwtklpec revk*cpfpsr pdngfvnypa kptlyykdka tfgchdgysl*
*241 dgpeeiectk lgnwsampsc k***asckvpvkk atvvyqgerv kiqekfkngm lhgdkvsffc**
301 knkekkcsyt edaqcidgti evpkcfkehs slafwktdas dvkpc

Italics: Domain 4

Bold: Domain 5

Underlined: identified sequence

References

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